

Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/100874/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Nomikos, Michail, Stamatiadis, Panagiotis, Sanders, Jessica Rose, Beck, Konrad ORCID: <https://orcid.org/0000-0001-5098-9484>, Calver, Brian Lewis, Buntwal, Luke, Lofty, Morgan, Sideratou, Zili, Swann, Karl ORCID: <https://orcid.org/0000-0002-4355-1449> and Lai, Francis Anthony ORCID: <https://orcid.org/0000-0003-2852-8547> 2017. Male infertility-linked point mutation reveals a vital binding role for the C2 domain of sperm PLC ζ . Biochemical Journal 474 (6), pp. 1003-1016. 10.1042/BCJ20161057 file

Publishers page: <http://dx.doi.org/10.1042/BCJ20161057>
<<http://dx.doi.org/10.1042/BCJ20161057>>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies.

See

<http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



Male infertility-linked point mutation reveals vital binding role for the C2 domain of sperm PLC ζ

Michail NOMIKOS^{*†1}, Panagiotis STAMATIADIS[†], Jessica SANDERS[†], Konrad BECK[‡],
Brian L. CALVER[†], Luke BUNTWAL[†], Morgan LOFTY[†], Zili SIDERATOU[#], Karl
SWANN[†], F. Anthony LAI^{†1}

^{*}College of Medicine, Qatar University, PO BOX 2713, Doha, Qatar

[†]College of Biomedical and Life Sciences, School of Biosciences, Cardiff University, Cardiff, UK

[‡]College of Biomedical and Life Sciences, School of Dentistry, Cardiff University, Cardiff, UK

[#]National Center for Scientific Research “Demokritos”, 15310 Aghia Paraskevi, Greece

¹ To whom correspondence should be addressed (email: lait@cf.ac.uk (FAL), mixosn@yahoo.com (MN)[^]).

[^]*Author for editorial correspondence prior to publication*

Short title: Discrete binding properties of sperm PLC ζ C2 domain

Abbreviations: phospholipase C-zeta, PLC ζ ; calcium, Ca²⁺; phosphatidylinositol 4,5-bisphosphate, PIP₂; inositol 1,4,5-trisphosphate, IP₃

Key words: Male infertility, Fertilization, Sperm, Phospholipase C zeta (PLC ζ), calcium oscillations, C2 domain

ABSTRACT

Sperm-specific phospholipase C zeta (PLC ζ) is widely considered to be the physiological stimulus that evokes intracellular calcium (Ca²⁺) oscillations that are essential for the initiation of egg activation during mammalian fertilization. A recent genetic study reported a male infertility case that was directly associated with a point mutation in PLC ζ C2 domain, where an isoleucine residue had been substituted with a phenylalanine (I489F). Here in, we have analysed the effect of this mutation on the *in vivo* Ca²⁺ oscillation-inducing activity and the *in vitro* biochemical properties of human PLC ζ . Microinjection of cRNA or recombinant protein corresponding to PLC ζ ^{I489F} mutant at physiological concentrations completely failed to cause Ca²⁺ oscillations and trigger development. However, this infertile phenotype could be effectively rescued by microinjection of relatively high (non-physiological) amounts of recombinant mutant PLC ζ ^{I489F} protein, leading to Ca²⁺ oscillations and egg activation. Our *in vitro* biochemical analysis suggested that PLC ζ ^{I489F} mutant displayed similar enzymatic properties but dramatically reduced binding to PI(3)P and PI(5)P-containing liposomes compared to wild-type PLC ζ . Our findings highlight the importance of PLC ζ at fertilization and the vital role of the C2 domain in PLC ζ function, possibly due to its novel binding characteristics.

INTRODUCTION

In mammalian oocytes (eggs), the fertilizing sperm evokes a series of pre-programmed biochemical and morphological events collectively known as 'egg activation'. It is now well established in all mammalian species studied, that the earliest step of egg activation involves marked increases in the levels of the egg cytosolic calcium concentration [Ca^{2+}], which are both necessary and sufficient for activation and early embryonic development [1-3]. Despite recent controversies, a gamete-specific phospholipase C (PLC) isoform, PLC ζ is widely considered as the physiological stimulus that induces the characteristic series of large cytoplasmic Ca^{2+} transients, known as Ca^{2+} oscillations, within the fertilizing oocyte [3-8]. Sperm PLC ζ is delivered from the fertilizing sperm into the egg cytoplasm, catalyses the hydrolysis of its membrane-bound phospholipid substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2), triggering the cytoplasmic Ca^{2+} oscillations through the inositol 1,4,5-trisphosphate (InsP_3) signaling pathway [3, 4, 9]. PLC ζ appears to be the smallest currently known mammalian PLC isozyme, with the most basic domain organization amongst all PLC isoforms. PLC ζ consists of four EF hand domains at the N-terminus, the characteristic X and Y catalytic domains in the centre, followed by a C-terminal C2 domain [4, 9]. Thus, PLC ζ possesses a similar domain organization to PLC $\delta 1$ with the remarkable exception that it lacks an N-terminal pleckstrin homology (PH) domain [9]. The notable lack of a PH domain, makes it unclear how this sperm-specific PLC isoform directly interacts and targets biological membranes. A recent report, suggested that the N-terminal lobe of the EF-hand domain of PLC ζ together with its positively charged XY-linker, has an essential role to provide a tether that facilitates proper PIP_2 substrate access and binding in the PLC ζ active site [10]. However, the exact mechanism that PLC ζ targets the PIP_2 -containing membrane is still unknown.

Evidence for the clinical importance of PLC ζ has been provided by studies that have directly linked reduced expression levels and abnormal forms of PLC ζ with male infertility [5, 11-15]. A very recent genetic study, using whole-exome sequencing analysis identified a homozygous missense mutation in the PLC ζ gene of two infertile brothers from Tunisia, presenting oocyte activation failure [8]. This mutation was located in the C2 domain of PLC ζ , where an isoleucine at position I489 had been replaced with a phenylalanine (I489F), (Fig. 1A) [8]. Interestingly, this is the first male infertility-linked PLC ζ point mutation to be reported within the C2 domain of PLC ζ , a domain which although is well known to be essential for PLC ζ function, its exact role still remains unclear. It was shown that I489F mutation results in

some loss of Ca^{2+} oscillation-inducing activity of PLC ζ in eggs, but the degree of loss of activity was not quantified and the poor ability of the PLC ζ mutant to trigger Ca^{2+} oscillations was not extensively characterized [8].

In the present study we introduced the infertility-linked PLC ζ I489F mutation into human PLC ζ sequence and we analysed the effect of this mutation on both the *in vivo* Ca^{2+} oscillation-inducing activity and the *in vitro* biochemical/enzymatic properties of human PLC ζ . For comparison, cRNA encoding luciferase-tagged versions of wild-type and PLC ζ^{I489F} mutant or bacterially-expressed recombinant proteins were microinjected into unfertilised mouse and bovine eggs. Circular Dichroism (CD) spectroscopy was used to investigate whether the I489F mutation interferes with the proper folding of the C2 domain. The enzymatic and biochemical properties of PLC ζ^{WT} and PLC ζ^{I489F} mutant were analyzed using an *in vitro* [^3H]PIP $_2$ hydrolysis and liposome binding assays.

MATERIALS AND METHODS

Plasmid construction

Human PLC ζ -luciferase in pCR3 vector [5] was subjected to site-directed mutagenesis (QuikChange II; Stratagene) to generate the PLC ζ^{I489F} mutant. PLC ζ^{WT} and PLC ζ^{I489F} mutant were amplified by PCR from the corresponding pCR3 plasmid using Phusion polymerase (Finnzymes) and the appropriate primers to incorporate a 5'-SalI site and a 3'-NotI site and were cloned into a modified pET expression vector (pETMM41). The primers used for the amplification of PLC ζ^{WT} and PLC ζ^{I489F} mutant were: 5'-CCTAGTCGACATGGAAATGAGATGGTTTTTGTGTC-3' (forward) and 5'-CTAAGCGGCCGCTCATCTGACGTACCAAACATAAAA-3' (reverse). Similarly to the full-length PLC ζ constructs, the C2 domains (480-608aa) of PLC ζ^{WT} and PLC ζ^{I489F} mutant were amplified by PCR from the aforementioned corresponding pCR3 plasmids using Phusion polymerase (Finnzymes) and the appropriate primers to incorporate a 5'-SalI site and a 3'-NotI site and were cloned into pETMM41 vector. The primers used for the amplification of C2 ζ^{WT} and C2 ζ^{I489F} mutant were: 5'-CACCGTCGACATGCCAATTACACTTACAATAAGG-3' (forward) and 5'-CTAAGCGGCCGCTCATCTGACGTACCAAACATAAAA-3' (reverse). Successful mutagenesis and cloning of the above constructs was confirmed by dideoxynucleotide sequencing (Applied Biosystems Big-Dye Version 3.1 chemistry and model 3730 automated capillary DNA sequencer by DNA Sequencing & ServicesTM).

cRNA synthesis

Luciferase-tagged PLC ζ^{WT} and PLC $\zeta^{1489\text{F}}$ constructs were linearized by restriction digests and then cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and a poly(A) tailing kit (Ambion), as per manufacturer instructions.

Protein expression and purification

E. coli (BL21-CodonPlus(DE3)-RILP; Stratagene) cells were transformed with the appropriate pETMM41 construct and cultured at 37°C until the A600 reached 0.6. Then protein expression was induced for 18 hours at 16°C with 0.1 mM isopropyl β -D-thiogalactopyranoside (IPTG; ForMedium). Induced cells were then harvested by centrifugation at 6,000 *g* for 10 minutes at 4°C and resuspended in ice-cold amylose column buffer [10 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM EDTA and protein inhibitor mixture (Roche)]. Then the resuspended cells were sonicated four times for 15 seconds on ice. After 20 minutes centrifugation at 20,000 *g* at 4°C, to remove the insoluble proteins, the soluble MBP-tagged fusion proteins were purified by affinity chromatography using amylose resin column following standard procedures (New England Biolabs). Eluted proteins were then dialyzed and concentrated using centrifugal concentrators (Sartorius; 10,000 molecular weight cut-off).

SDS-PAGE and Western blot

Recombinant MBP fusion protein were separated by SDS-PAGE and immunoblot analysis was performed as described previously [10]. Proteins were probed with a monoclonal penta-His antibody (1:5,000 dilution).

Preparation and handling of mouse oocytes

Mature MII oocytes (eggs) were collected from female MF1 mice (Envigo Ltd) of 6-8 weeks old, 15 hours after injection with 10 IU human chorionic gonadotrophin (hCG). Approximately 48 hours before hCG injection mice were injected with 10 IU pregnant mare's serum gonadotrophin. Following collection, cumulus cells were removed using hyaluronidase treatment and eggs were maintained in M2 media (Sigma Aldrich) under mineral oil at 37°C until use. Injected eggs were transferred for development in KSOM Media (Embryomax by Millipore). All animal work was conducted according to Home Office Licensing procedures and approved by the Animals Ethics Committee at Cardiff University.

Preparation and handling of bovine oocytes

Ovaries were collected after slaughter from the local abattoir and transported to the laboratory at 24 °C in PBS (Sigma P4417) within 1 hour. Cumulus oocyte complexes were collected by slashing the surface of the ovary with a scalpel in Medium 199 – Hepes buffered and the solution was passed through a 100-micron mesh filter in order to retain the cumulus oocyte complexes (COC). COC were placed in maturation media for 22h [16]. After completion of maturation COC were vortexed for 3-4 min in hyaluronidase in order to isolate the mature eggs, which were immediately washed and transferred in M2 media until use.

Protein microinjection and measurements of intracellular Ca^{2+}

Eggs were incubated in M2 media containing Cal-520 AM (5 μM) for 30 minutes at 37 °C before the experiment. Eggs were held in M2 for microinjection [17]. Recombinant $\text{PLC}\zeta^{\text{WT}}$ and $\text{PLC}\zeta^{\text{I489F}}$ mutant fusion proteins were diluted in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4) prior to introduction into oocytes using a high pressure injection method. Alexa Fluor 594 (1 mM) was used as a loading control to ensure the equal protein volume injection. Eggs were imaged in HKSOM media using a Nikon TE2000 inverted epifluorescence microscope connected to a cooled intensified CCD camera (Photek,UK) and fluorescence was recorded by photon counting software (Photek,UK). In cases where luciferase expression was measured the fluorescence (to quantify Ca^{2+} changes) was recorded alternately using a 10 second switching cycle [18]. The fluorescence signal was normalised to relative fluorescence by plotting absolute fluorescence divided by basal level fluorescence (F/F_0). All egg experiments were conducted within a three (3) week period.

cRNA microinjection and measurements of intracellular Ca^{2+} and luciferase expression

Eggs were held in M2 for microinjection. cRNA was diluted in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4) and in the case of cRNA also mixed with 1 mM Oregon Green BAPTA dextran OGBD (Life Technologies) prior to introduction into oocytes using a high pressure injection method [17]. Bolus injection calculated the amount of injection solution microinjected that was approximately 3-5% of oocyte volume. Eggs were imaged in HKSOM media containing 100 μM luciferin using a Nikon TE2000 inverted epifluorescence microscope connected to a cooled intensified CCD camera (Photek,UK) and both luminescence and fluorescence were recorded by photon counting software (Photek, UK). Luminescence (quantifying luciferase expression) and fluorescence (quantifying Ca^{2+} changes) were recorded alternately using a 10 second switching cycle [18] with these 2 signals being plotted

individually for each oocyte over the same time scale. The fluorescence signal was normalised to relative fluorescence by plotting absolute fluorescence divided by basal level fluorescence (F/F_0) and luminescence was plotted as a running average over 5 minutes. All egg experiments were conducted within a 3-month period.

CD spectroscopy

CD spectra of MBP, MBP-C2 ζ^{WT} and MBP-C2 ζ^{I489F} were recorded on an Aviv model 215 instrument (Aviv Biomedical Inc., Lakewood, NJ) using a 0.1-cm quartz cell at 4 °C. Proteins were dissolved in 100 mM NaF, 20 mM KH₂PO₄/NaOH, pH 7.0, at a concentration of ~ 0.15 mg/ml. Concentrations were determined based on the absorbance at 280 nm assuming extinction coefficients derived from the amino acid composition [19]. Secondary structure content was analysed using the CDstr algorithm [20] as implemented on DichroWeb [21] using the SMP180 reference spectra [22].

Thermal stability was monitored at 221nm in 0.5 °C intervals from 4 °C to 70 °C (MBP) or up to a maximum temperature when protein aggregation was observed as indicated by a sharp increase of the dynode voltage with settings resulting in an average heating rate of ~ 30 °C/h. Apparent melting temperatures T_m and van't Hoff's enthalpies ΔH_{vH} were estimated from non-linear curve fitting assuming a 2-state folded-to-unfolded transition as described [23] with the ellipticity of the unfolded state set as that observed for MBP.

Molecular modelling

Structural models of MBP, MBP-C2 ζ^{WT} and MBP-C2 ζ^{I489F} were generated using SWISS-MODEL with the corresponding parts of the PDB coordinates 3mq9 (residues 5 to 366) and 1djg (rat PLC δ 1, residues 496 to 624) as templates [24].

PIP₂ hydrolysis assays

The PIP₂ hydrolytic activity of recombinant MBP-tagged PLC ζ proteins was determined as described previously [5, 10, 25]. The final PIP₂ concentration in the reaction mixture was 220 μM , containing 0.05 μCi of [³H]PIP₂. For the assays examining the Ca²⁺ dependence of PLC enzymatic activity, the Ca²⁺ buffers were prepared by EGTA/CaCl₂ admixture, while in assays to determine the dependence on substrate PIP₂ concentration, 0.05 μCi of [³H]PIP₂ was mixed with cold PIP₂ to give the appropriate final concentration [10, 26].

K_m and EC_{50} values of Ca^{2+} dependence for PIP_2 hydrolysis for the MBP-tagged $PLC\zeta$ recombinant proteins were determined by non-linear regression analysis (GraphPad Prism 5).

Liposome preparation and binding assays.

Unilamellar liposomes were prepared as previously described [10, 26]. For the protein-liposome binding experiments, liposomes (100 μ g) were incubated with 1 μ g of each MBP-tagged recombinant protein for 30 min at room temperature and centrifuged for 5 hours at 4 °C. Supernatants and pellets were analysed either by SDS-PAGE and Coomassie Brilliant Blue staining or by the [3H] PIP_2 hydrolysis assay, as previously described [10].

RESULTS

Microinjection of cRNA encoding $PLC\zeta^{I489F}$ mutant completely failed to trigger Ca^{2+} oscillations when expressed at physiological concentrations in mouse eggs

To investigate the impact of I489F mutation on the *in vivo* Ca^{2+} oscillation-inducing activity of human $PLC\zeta$, we used site-directed mutagenesis to generate the $PLC\zeta^{I489F}$ mutant. To enable direct comparative analysis with $PLC\zeta^{WT}$ and to verify that this construct was faithfully expressed as protein in cRNA-microinjected unfertilized mouse eggs, we produced this mutant as a luciferase-fusion construct, as previously described [10, 26, 27]. Microinjection of cRNA encoding luciferase-tagged $PLC\zeta^{WT}$ ($PLC\zeta^{WT}$ -LUC) caused prominent Ca^{2+} oscillations in all injected eggs (~4.0 spikes in the first hour of oscillating), similar to those observed during fertilization, following successful protein expression to a level indicated by a luminescence reading of 0.06 c.p.s., (Fig. 1B left panel, Table 1). In contrast, microinjection of cRNA corresponding to $PLC\zeta^{I489F}$ -LUC mutant failed to cause any Ca^{2+} oscillations at equivalent protein expression levels (0.06 c.p.s.), (Fig. 1B right panel, Table 1). Interestingly, expression of $PLC\zeta^{I489F}$ -LUC mutant at significantly higher levels (0.74 c.p.s.) led to low frequency Ca^{2+} oscillations (~2.3 spikes in the first hour of oscillating), (Fig. 1C, Table 1), suggesting that overexpression of $PLC\zeta^{I489F}$ -LUC, which leads to overload of this mutant within the egg cytoplasm, can induce Ca^{2+} release from intracellular stores.

Microinjection of MBP- $PLC\zeta^{I489F}$ protein is less effective at triggering Ca^{2+} oscillations in mouse eggs

PLC ζ ^{WT} and PLC ζ ^{I489F} mutant were then subcloned into the pETMM41 expression vector to allow prokaryotic expression of these constructs, as 6xHis-MBP-fusion recombinant proteins. We have previously demonstrated that NusA is a powerful fusion partner for PLC ζ , significantly enhancing the bacterial expression, protein solubility, as well as the purified recombinant protein stability over time [5, 10]. In this study, we demonstrate for first time that in addition to NusA, MBP tag is an extremely effective protein tag for PLC ζ . Our comparative experiments with NusA tag showed that MBP is an effective protein fusion partner for PLC ζ preserving its stability over time (data not shown).

Optimal protein production for these MBP-fusion PLC ζ constructs required induction of protein expression with 0.1 mM IPTG for 18 h at 16 °C. Following bacterial expression in *E. coli* and isolation by amylose resin affinity chromatography, the purified recombinant proteins were analysed by SDS-PAGE and immunoblot analysis using an anti-His (penta-His) monoclonal antibody (Fig. 2A). The dominant protein band with mobility corresponding to the predicted molecular mass for MBP-PLC ζ ^{WT} and MBP-PLC ζ ^{I489F} mutant was observed for both recombinant fusion proteins (~118 kDa). These major bands were also confirmed by immunoblot analysis by the penta-His antibody (Fig. 2A right panel). Some additional, fainter, low molecular weight bands could be observed, which were also detected by the penta-His antibody and were probably the result of protease degradation occurring during the various stages of protein expression and purification. Microinjection of MBP-PLC ζ ^{WT} into mouse eggs at a concentration of 0.0375 mg/ml revealed that it possesses a potent ability to trigger cytoplasmic Ca²⁺ oscillations (Fig. 2B; upper panel), matching that observed after microinjection of native sperm extracts [28]. Microinjection of the MBP protein alone was unable to induce Ca²⁺ release. Interestingly, microinjection of MBP-PLC ζ ^{I489F} mutant protein at equivalent levels that MBP-PLC ζ ^{WT} triggered physiological Ca²⁺ oscillations (0.0375 mg/ml), was either unable to induce any Ca²⁺ oscillations (13/23 eggs) or could only trigger very low frequency Ca²⁺ oscillations (10/23 eggs), (Fig. 2B; lower panel).

To investigate whether it was possible to rescue the low frequency Ca²⁺ oscillations, MBP-PLC ζ ^{I489F} recombinant protein was microinjected at higher levels. We found that a 2-fold increase in the amount of MBP-PLC ζ ^{I489F} mutant (0.075 mg/ml) microinjected into mouse eggs was able to rescue the defective Ca²⁺ oscillation-inducing phenotype and trigger egg activation, as indicated by cleavage of the 2-cell stage (Fig. 3). These findings agree with our previous observations, suggesting that overload of PLC ζ ^{I489F} mutant within the mouse egg cytoplasm, can induce Ca²⁺ oscillations.

MBP-PLC ζ ^{WT} protein is more potent in triggering Ca²⁺ oscillations in bovine eggs than MBP-PLC ζ ^{I489F}

To examine whether our previous observations regarding the ability of PLC ζ ^{I489F} mutant to trigger Ca²⁺ oscillations in mouse eggs are consistent in eggs of a different species, we compared the abilities of MBP-PLC ζ ^{WT} and MBP-PLC ζ ^{I489F} recombinant proteins to induce Ca²⁺ oscillations in bovine eggs. The optimal concentration for MBP-PLC ζ ^{WT} to induce a physiological pattern of Ca²⁺ oscillations in bovine eggs was 0.15 mg/ml. In contrast, microinjection of MBP-PLC ζ ^{I489F} mutant protein at this concentration was able to trigger very low frequency Ca²⁺ oscillations in all microinjected eggs (Fig. 4).

I489F does not alter the folding and the thermal stability of PLC ζ C2 domain

To investigate whether I489F mutation within the C2 domain of PLC ζ interferes with the proper folding of this domain, we analysed the MBP-tagged wild type and mutant C2 domains (C2 ζ ^{WT} and C2 ζ ^{I489F}) by CD spectroscopy. Attempts to produce an untagged or 6xHis-tag version of the C2 domain of PLC ζ ^{WT} using the bacterial expression system proved unsuccessful, as the protein appeared to be completely insoluble, accumulating into inclusion bodies. Thus, C2 ζ ^{WT} and C2 ζ ^{I489F} (Fig. 5A) were cloned into pETMM41 expression vector to allow bacterial expression of these domains as 6xHis-MBP-fusion recombinant proteins. The presence of MBP tag significantly enhanced the expression of soluble C2 domains and the affinity-purified MBP-tagged C2 domains after SDS-PAGE and immunoblot analysis using the penta-His antibody, displayed the predicted molecular mass (~59 kDa); (Fig. 5B). It is worth noting, that prior the CD experiments removal of the MBP moiety from the C2 domains was attempted but this resulted in rapid degradation of the proteins. Thus, the intact MPB-C2 domains were used for our CD studies, while the MBP moiety alone served as a control for our experiments. Spectra recorded at 4 °C were indistinguishable from each other, but different from that of MBP alone (Fig. 6A). Deconvolution of the spectra resulted in 39 % α helix / 18 % β strand and 35 % α helix / 24 % β strand for MBP by itself and the two fusion proteins, respectively. These values are in very good agreement with those of homology based models of MBP (41 % α helix, 17 % β strand) and a PLC ζ C2 domain combined with MBP (35 % α helix, 27 % β strand). The thermal stability was measured by monitoring the CD signal at 221nm (Fig. 6B). All samples showed a steep decrease in ellipticity. In contrast to MBP, the fusion proteins precipitated upon unfolding as indicated by an increase of light scattering.

Assuming a two-state folded-to-unfolded transition and extrapolating to the ellipticity observed for unfolded MBP, fitting of the data resulted in melting temperature $T_m = 60.5 \pm 0.5$ °C and van't Hoff's enthalpies of ~560 and ~250 kJ/mol for MBP and the two fusion proteins, respectively. For MBP, these values agree with those previously reported [29].

I489F does not alter the *in vitro* enzymatic properties of human PLC ζ

We then examined the impact of I489F mutation on the *in vitro* enzymatic properties of PLC ζ . To determine the specific PIP₂ hydrolytic enzyme activities for PLC ζ^{WT} and PLC ζ^{I489F} a micellar [³H]PIP₂ hydrolysis assay was used as previously described [10, 27, 30]. The histogram of Fig. 7A and Table 2 reveal that the enzymatic activities of PLC ζ^{WT} and PLC ζ^{I489F} were almost identical (960±43 vs 953±49 nmol/min/mg), suggesting that I489F mutation has no effect on the ability of PLC ζ to hydrolyse *in vitro* PIP₂. In order, to investigate the effect of I489F mutation on Ca²⁺ sensitivity of PLC ζ enzyme activity we assessed the ability of PLC ζ^{WT} and PLC ζ^{I489F} to hydrolyse [³H]PIP₂ at different Ca²⁺ concentrations ranging from 0.1 nM to 0.1 mM. The resulting EC₅₀ values for PLC ζ^{WT} (66 nM) and PLC ζ^{I489F} (60 nM) were very similar (Fig. 7B, Table 2). In addition, calculation of the Michaelis-Menten constant *K_m*, for PLC ζ^{WT} and PLC ζ^{I489F} also yielded in comparable values (81 vs 93 μM), (Table 2), indicating that I489F mutation has no effect on the Ca²⁺ sensitivity or on the *in vitro* enzymatic affinity of PLC ζ for its substrate, PIP₂.

I489F dramatically reduces the binding of PLC ζ to PI(3)P and PI(5)P

The only specific binding partners for PLC ζ C2 domain that have been *in vitro* identified up to date are PI(3)P and PI(5)P [31, 32]. To examine the binding properties of PLC ζ^{I489F} to PIP₂, PI(3)P and PI(5)P we employed two different approaches, a liposome-binding (pull-down) and a liposome-binding/enzyme assay, as previously described [10, 26]. For these experiments, we prepared unilamellar liposomes, which were composed of PC:CHOL:PE (4:2:1) with incorporation of either 1% PIP₂, 5% PI(3)P or 5% PI(5)P. For diminishing the non-specific protein binding to highly charged lipids, the liposome binding experiments were performed in the presence of a near-physiological concentration of MgCl₂ (0.5mM) [26]. The MBP moiety alone served as the negative control for our experiments. As shown in Fig. 8, MBP did not exhibit any specific liposome binding in the absence or presence of PIP₂, PI(3)P and PI(5)P, whereas our positive control MBP-PLC ζ^{WT} displayed robust binding to liposomes containing either 1% PIP₂, 5% PI(3)P or 5% PI(5)P. On the other hand,

MBP-C2 ζ^{WT} showed significant binding only to liposomes containing either 5% PI(3)P or 5% PI(5)P. In contrast, although MBP-PLC ζ^{I489F} was able to bind strongly to liposomes containing 1% PIP₂, its binding to liposomes containing either 5% PI(3)P or 5% PI(5)P significantly diminished (Fig. 8). Similarly, MBP-C2 ζ^{I489F} was unable to bind liposomes containing 5% PI(3)P or 5% PI(5)P. These findings clearly suggest that I489F mutation clearly affects the binding of PLC ζ C2 domain to PI(3)P and PI(5)P. For more quantitative analysis, a liposome-binding/enzyme assay, was employed to analyze the binding of MBP-PLC ζ^{WT} and MBP-PLC ζ^{I489F} to PIP₂, PI(3)P and PI(5)P. Thus, 1 μg of recombinant protein corresponding to MBP-PLC ζ^{WT} and MBP-PLC ζ^{I489F} were incubated with liposomes containing either 1% PIP₂, 5% PI(3)P or 5% PI(5)P. After centrifugation, the supernatant were separated for the precipitated liposomes and the PIP₂ hydrolytic activity determined using the standard [³H]PIP₂ hydrolysis assay. Based on the % of the PIP₂ hydrolytic activity pre- and post-liposome binding, we estimated the relative binding of each PLC ζ protein to the different phosphoinositide specific-containing liposomes [10]. As shown at histograms in Fig. 9, the binding of PLC ζ^{WT} and PLC ζ^{I489F} to PIP₂-containing liposomes was almost identical, while the binding of PLC ζ^{I489F} to PI(3)P- and PI(5)P-containing liposomes had been reduced by ~50% compared to PLC ζ^{WT} , suggesting that I489F mutation indeed dramatically reduces the binding of PLC ζ to PI(3)P and PI(5)P.

DISCUSSION

Mounting experimental and clinical evidence strongly supports the notion that sperm-specific PLC ζ is the sole physiological stimulus of egg activation during mammalian fertilization [4, 8, 11-14, 33-36]. Sperm-delivered PLC ζ triggers the repetitive Ca²⁺ oscillations within the fertilized egg, by catalysing the hydrolysis of PIP₂ stimulating the InsP₃ signalling pathway [3, 9]. Although PLC ζ is the smallest PLC isoform with the most basic domain structure organization, its discrete biochemical properties contribute to its supreme effectiveness in triggering the Ca²⁺ signalling phenomenon within the fertilized mammalian eggs [9].

The past few years, clinical reports have directly linked defects in human PLC ζ with documented cases of male infertility. Firstly, Yoon et al., [11] reported a number of infertile patients presenting oocyte activation failure, providing evidence that was due to absence of reduced levels of PLC ζ within their sperm [11]. Then, the first direct link between male

infertility and a mutation on PLC ζ gene came from a study that reported a mutation on the catalytic domain of PLC ζ (H398P) of a patient who failed fertilization after ICSI [12]. Interestingly, another study reported a second PLC ζ mutation on the same heterozygous infertile patient, also in the catalytic domain (H233L) [14]. Recently, Escoffier et al., [8] reported a homozygous missense mutation in the PLC ζ gene of two infertile brothers from Tunisia, presenting egg activation failure [8]. This mutation was located in the C2 domain of PLC ζ where an Ile had been replaced with a Phe residue (I489F), (Fig. 1A) making it the first PLC ζ mutation located in a different domain than the catalytic.

Here, we introduced the infertility-linked I489F PLC ζ mutation in human PLC ζ sequence and we assessed the effects of this mutation upon the *in vivo* Ca²⁺ oscillation-inducing activity and the *in vitro* biochemical and enzymatic properties of human PLC ζ . Our study provides evidence and extends the previous work of Escoffier et al., [8] by revealing that (i) PLC ζ I489F mutation dramatically reduces the Ca²⁺ oscillation-inducing activity of PLC ζ in mouse and bovine eggs (Figs 1B, 2B, 4). Although microinjection of physiological levels of PLC ζ mutant are either unable or triggered very low frequency Ca²⁺ oscillations in mouse and bovine eggs, a 2-fold increase in the amount of PLC ζ microinjected in mouse eggs was capable of rescuing the defective Ca²⁺ oscillation-inducing phenotype, triggering egg activation of these PLC ζ ^{I489F} microinjected eggs (Fig. 3). This suggests that by overloading the egg with significantly higher amounts of this PLC ζ mutant can lead to successful oocyte activation, explaining the infertility of the two heterozygous brothers carrying this mutation. (ii) CD spectroscopy showed that the I489F C2 mutation has no effect on the proper folding and the thermal stability of this domain (Fig. 6). This was consistent with our observations regarding the enzymatic properties of PLC ζ ^{I489F} mutant, which were almost identical with the enzymatic properties of PLC ζ ^{WT} (Fig. 7, Table 2), suggesting that I489F mutation has no effect on the ability of PLC ζ to hydrolyse PIP₂. (iii) More importantly, our liposome binding experiments revealed that I489F mutation dramatically reduces (~50%) the binding of PLC ζ to PI(3)P and PI(5)P (Figs. 8, 9), two phosphoinositides, which have been previously reported to interact with PLC ζ *in vitro* [31, 32]. To the best of our knowledge, PLC ζ C2 domain is the first C2 domain amongst the C2 domains of all PLC isoforms that it has been shown to directly interact with PI(3)P and PI(5)P, *in vitro*. Although it is difficult to predict which amino acid residues play a role on these interactions, we have shown that I489 is a key residue for efficient binding of PLC ζ to both PI(3)P and PI(5)P. High resolution three-dimensional structure analysis of PLC ζ

C2 domain by X-ray crystallography could help to reveal the critical binding sites for these interactions. It is now also necessary to understand the physiological role of the binding of PLC ζ to PI(3)P and PI(5)P and also to extensively search for any other unidentified membrane egg proteins, which might interact with the C2 domain of PLC ζ , assisting with the proper localization and targeting of this enzyme within the egg cytoplasm.

It has been proposed, at least for mouse PLC ζ , that its nuclear translocation ability regulates the cell-cycle dependent Ca²⁺ oscillations [3]. It is worth noting that there is no scientific evidence for the C2 domain playing a role in the nuclear sequestration of PLC ζ , as it has been clearly demonstrated that the nuclear localization signal (NLS) of mouse PLC ζ is located within the XY linker region, close to the start of the Y domain [3].

Understanding the complex mechanism of action of PLC ζ requires further investigation. However, based on our previous and recent findings we propose that after sperm-egg fusion and the delivery of PLC ζ from the sperm into the egg cytoplasm, the high Ca²⁺ sensitivity of this prototypic PLC is conferred by its EF hand domains, allowing it to be active at resting egg Ca²⁺ levels [27, 31, 37]. PLC ζ may then associate with a specific intracellular membrane by interaction of the C2 domain with either PI(3)P, PI(5)P or another unidentified egg membrane protein. Finally, the positively charged XY linker together with the first EF hand domain provide a tether to facilitate proper PIP₂ substrate access, in order the catalytic XY domain to proceed with the catalysis of the hydrolysis of PIP₂ to produce InsP₃ (Fig. 10) [10].

In conclusion, the identification of the first male infertility-linked PLC ζ point mutation located in the C2 domain of PLC ζ , provides the first clinical support for the vital role of this domain on PLC ζ function. As our study proposes, this is purely due to its novel binding properties of this domain to PI(3)P and PI(5)P or other unidentified egg factor. In addition, the identification of another male infertility-linked PLC ζ mutation necessitates the use of recombinant PLC ζ protein in a clinical setting with the aim to rescue such cases of oocyte activation failure.

AUTHOR CONTRIBUTIONS

M.N., K.S. and F.A.L. devised the project strategy. M.N., K.B., K.S. and F.A.L. designed the experiments, which were performed by M.N., P.S., J.S., K.B., B.L.C., L.B., M.L.,

and Z.S. M.N. prepared the first manuscript draft, which was revised and approved by all authors.

FUNDING

This work was supported by an EU-FP7 Marie-Curie Intra-European Fellowship 628634 (to M.N.), an Institute for Molecular and Experimental Medicine Research Scholarship (to J.R.S.) and partially by a research grant from Cook Medical Technologies (to K.S. and F.A.L.). F.A.L and K.S. hold patents on PLC ζ with Cardiff University.

DECLARATIONS OF INTEREST: The authors declare no financial interests.

REFERENCES

- 1 Stricker, S. A. (1999) Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol.* **211**, 157-176
- 2 Runft, L. L., Jaffe, L. A. and Mehlmann, L. M. (2002) Egg activation at fertilization: where it all begins. *Dev Biol.* **245**, 237-254
- 3 Nomikos, M., Kashir, J., Swann, K. and Lai, F. A. (2013) Sperm PLCzeta: from structure to Ca²⁺ oscillations, egg activation and therapeutic potential. *FEBS Lett.* **587**, 3609-3616
- 4 Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K. and Lai, F. A. (2002) PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development.* **129**, 3533-3544
- 5 Nomikos, M., Yu, Y., Elgmati, K., Theodoridou, M., Campbell, K., Vassilakopoulou, V., Zikos, C., Livanou, E., Amso, N., Nounesis, G., Swann, K. and Lai, F. A. (2013) Phospholipase Czeta rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril.* **99**, 76-85
- 6 Nomikos, M., Sanders, J. R., Kashir, J., Sanusi, R., Buntwal, L., Love, D., Ashley, P., Sanders, D., Knaggs, P., Bunkheila, A., Swann, K. and Lai, F. A. (2015) Functional disparity between human PAWP and PLCzeta in the generation of Ca²⁺ oscillations for oocyte activation. *Mol Hum Reprod.* **21**, 702-710
- 7 Satouh, Y., Nozawa, K. and Ikawa, M. (2015) Sperm postacrosomal WW domain-binding protein is not required for mouse egg activation. *Biol Reprod.* **93**, 94
- 8 Escoffier, J., Lee, H. C., Yassine, S., Zouari, R., Martinez, G., Karaouzene, T., Coutton, C., Kherraf, Z. E., Halouani, L., Triki, C., Nef, S., Thierry-Mieg, N., Savinov, S. N., Fissore, R., Ray, P. F. and Arnoult, C. (2016) Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. *Hum Mol Genet.* **25**, 878-891
- 9 Nomikos, M. (2015) Novel signalling mechanism and clinical applications of sperm-specific PLCzeta. *Biochem Soc Trans.* **43**, 371-376
- 10 Nomikos, M., Sanders, J. R., Parthimos, D., Buntwal, L., Calver, B. L., Stamatiadis, P., Smith, A., Clue, M., Sideratou, Z., Swann, K. and Lai, F. A. (2015) Essential Role of the EF-hand Domain in Targeting Sperm Phospholipase Czeta to Membrane Phosphatidylinositol 4,5-Bisphosphate (PIP2). *J Biol Chem.* **290**, 29519-29530
- 11 Yoon, S. Y., Jellerette, T., Salicioni, A. M., Lee, H. C., Yoo, M. S., Coward, K., Parrington, J., Grow, D., Cibelli, J. B., Visconti, P. E., Mager, J. and Fissore, R. A. (2008) Human sperm devoid of PLC, zeta 1 fail to induce Ca(2+) release and are unable to initiate the first step of embryo development. *J Clin Invest.* **118**, 3671-3681

- 12 Heytens, E., Parrington, J., Coward, K., Young, C., Lambrecht, S., Yoon, S. Y., Fissore, R. A., Hamer, R., Deane, C. M., Ruas, M., Grasa, P., Soleimani, R., Cuvelier, C. A., Gerris, J., Dhont, M., Deforce, D., Leybaert, L. and De Sutter, P. (2009) Reduced amounts and abnormal forms of phospholipase C zeta (PLCzeta) in spermatozoa from infertile men. *Hum Reprod.* **24**, 2417-2428
- 13 Kashir, J., Heindryckx, B., Jones, C., De Sutter, P., Parrington, J. and Coward, K. (2010) Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update.* **16**, 690-703
- 14 Kashir, J., Konstantinidis, M., Jones, C., Lemmon, B., Lee, H. C., Hamer, R., Heindryckx, B., Deane, C. M., De Sutter, P., Fissore, R. A., Parrington, J., Wells, D. and Coward, K. (2012) A maternally inherited autosomal point mutation in human phospholipase C zeta (PLCzeta) leads to male infertility. *Hum Reprod.* **27**, 222-231
- 15 Nomikos, M., Elgmami, K., Theodoridou, M., Calver, B. L., Cumbes, B., Nounesis, G., Swann, K. and Lai, F. A. (2011) Male infertility-linked point mutation disrupts the Ca²⁺ oscillation-inducing and PIP(2) hydrolysis activity of sperm PLCzeta. *Biochem J.* **434**, 211-217
- 16 Chung, J. T., Keefer, C. L. and Downey, B. R. (2000) Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI). *Theriogenology.* **53**, 1273-1284
- 17 Swann, K., Campbell, K., Yu, Y., Saunders, C. and Lai, F. A. (2009) Use of luciferase chimera to monitor PLCzeta expression in mouse eggs. *Methods Mol Biol.* **518**, 17-29
- 18 Campbell, K. and Swann, K. (2006) Ca²⁺ oscillations stimulate an ATP increase during fertilization of mouse eggs. *Dev Biol.* **298**, 225-233
- 19 Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**, 2411-2423
- 20 Johnson, W. C. (1999) Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins.* **35**, 307-312
- 21 Whitmore, L. and Wallace, B. A. (2008) Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. *Biopolymers.* **89**, 392-400
- 22 Abdul-Gader, A., Miles, A. J. and Wallace, B. A. (2011) A reference dataset for the analyses of membrane protein secondary structures and transmembrane residues using circular dichroism spectroscopy. *Bioinformatics.* **27**, 1630-1636
- 23 Greenfield, N. J. (2004) Analysis of circular dichroism data. *Methods Enzymol.* **383**, 282-317
- 24 Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Gallo Cassarino, T., Bertoni, M., Bordoli, L. and Schwede, T. (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* **42**, W252-258
- 25 Nomikos, M., Elgmami, K., Theodoridou, M., Georgilis, A., Gonzalez-Garcia, J. R., Nounesis, G., Swann, K. and Lai, F. A. (2011) Novel regulation of PLCzeta activity via its XY-linker. *Biochem J.* **438**, 427-432
- 26 Theodoridou, M., Nomikos, M., Parthimos, D., Gonzalez-Garcia, J. R., Elgmami, K., Calver, B. L., Sideratou, Z., Nounesis, G., Swann, K. and Lai, F. A. (2013) Chimeras of sperm PLCzeta reveal disparate protein domain functions in the generation of intracellular Ca²⁺ oscillations in mammalian eggs at fertilization. *Mol Hum Reprod.* **19**, 852-864
- 27 Nomikos, M., Blayney, L. M., Larman, M. G., Campbell, K., Rossbach, A., Saunders, C. M., Swann, K. and Lai, F. A. (2005) Role of phospholipase C-zeta domains in Ca²⁺-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca²⁺ oscillations. *J Biol Chem.* **280**, 31011-31018
- 28 Swann, K. and Yu, Y. (2008) The dynamics of calcium oscillations that activate mammalian eggs. *Int J Dev Biol.* **52**, 585-594
- 29 Beena, K., Udgaonkar, J. B. and Varadarajan, R. (2004) Effect of signal peptide on the stability and folding kinetics of maltose binding protein. *Biochemistry.* **43**, 3608-3619
- 30 Nomikos, M., Theodoridou, M., Elgmami, K., Parthimos, D., Calver, B. L., Buntwal, L., Nounesis, G., Swann, K. and Lai, F. A. (2014) Human PLCzeta exhibits superior fertilization potency over mouse PLCzeta in triggering the Ca(2+) oscillations required for mammalian oocyte activation. *Mol Hum Reprod.* **20**, 489-498

- 31 Kouchi, Z., Shikano, T., Nakamura, Y., Shirakawa, H., Fukami, K. and Miyazaki, S. (2005) The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase C ζ . *J Biol Chem.* **280**, 21015-21021
- 32 Nomikos, M., Elgmati, K., Theodoridou, M., Calver, B. L., Nounesis, G., Swann, K. and Lai, F. A. (2011) Phospholipase C ζ binding to PtdIns(4,5)P₂ requires the XY-linker region. *J Cell Sci.* **124**, 2582-2590
- 33 Cox, L. J., Larman, M. G., Saunders, C. M., Hashimoto, K., Swann, K. and Lai, F. A. (2002) Sperm phospholipase C ζ from humans and cynomolgus monkeys triggers Ca²⁺ oscillations, activation and development of mouse oocytes. *Reproduction.* **124**, 611-623
- 34 Knott, J. G., Kurokawa, M., Fissore, R. A., Schultz, R. M. and Williams, C. J. (2005) Transgenic RNA interference reveals role for mouse sperm phospholipase C ζ in triggering Ca²⁺ oscillations during fertilization. *Biol Reprod.* **72**, 992-996
- 35 Kashir, J., Nomikos, M., Lai, F. A. and Swann, K. (2014) Sperm-induced Ca²⁺ release during egg activation in mammals. *Biochem Biophys Res Commun.* **450**, 1204-1211
- 36 Amdani, S. N., Yeste, M., Jones, C. and Coward, K. (2016) Phospholipase C ζ (PLC ζ) and male infertility: Clinical update and topical developments. *Adv Biol Regul.* **61**, 58-67
- 37 Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T. and Miyazaki, S. (2004) Recombinant phospholipase C ζ has high Ca²⁺ sensitivity and induces Ca²⁺ oscillations in mouse eggs. *J Biol Chem.* **279**, 10408-10412

TABLE LEGENDS

Table 1

Properties of luciferase-tagged PLC ζ^{WT} and PLC ζ^{I489F} mutant expressed in unfertilised mouse eggs

Ca²⁺-oscillation-inducing activity and luciferase luminescence levels (cps) in the 1st hour of spiking are summarized for mouse eggs microinjected with luciferase-tagged PLC ζ^{WT} and PLC ζ^{I489F} mutant. Results are expressed as means \pm S.E.M.

Table 2

***In vitro* enzymatic properties of MBP-tagged PLC ζ^{WT} and PLC ζ^{I489F} mutant**

Summary of specific enzyme activity, *K_m* and EC₅₀ values of Ca²⁺-dependence for PIP₂ hydrolysis, determined by non-linear regression analysis (GraphPad Prism 5), (see Fig. 7).

FIGURE LEGENDS

Figure 1

Effect of I489F mutation on Ca^{2+} -oscillation-inducing activity of human PLC ζ in mouse eggs

(A) Schematic representation of human PLC ζ domain organisation identifying the location of I489F mutation within the C-terminal C2 domain. (B, C) Fluorescence and luminescence recordings reporting the Ca^{2+} changes [fluorescence (black traces) and luciferase expression (red traces; luminescence) in cps respectively] in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged PLC ζ^{WT} and PLC ζ^{I489F} mutant (see also Table 1).

Figure 2

MBP-PLC ζ^{I489F} recombinant protein fails to induce physiological pattern of Ca^{2+} oscillations in mouse eggs, when microinjected in equivalent concentration levels to MBP-PLC ζ^{WT}

(A) SDS-PAGE of affinity-purified recombinant MBP-tagged PLC ζ^{WT} and PLC ζ^{I489F} proteins (2 μg) analysed by 8% SDS-PAGE and Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using the penta-His antibody (1:5,000 dilution); (right panel). (B) Patterns of Ca^{2+} oscillations in unfertilized mouse eggs following microinjection of MBP-PLC ζ^{WT} and MBP-PLC ζ^{I489F} recombinant proteins. F/F0 represents fluorescent intensity of Cal520 relative to baseline.

Figure 3

Microinjection of higher (non-physiological) levels of MBP-PLC ζ^{I489F} recombinant protein in mouse eggs induces physiological pattern of Ca^{2+} oscillations and triggers egg activation.

F/F0 represents fluorescent intensity relative to baseline. The right panel shows 2-cell stage mouse embryos 22-24h after injection of MBP-PLC ζ^{I489F} recombinant protein.

Figure 4

MBP-PLC ζ ^{WT} recombinant protein has greater potency in triggering Ca²⁺ oscillations in bovine eggs compared to MBP-PLC ζ ^{I489F}

Bovine eggs were injected with MBP-PLC ζ ^{WT} recombinant protein (top trace) or MBP-PLC ζ ^{I489F} protein (bottom trace) and Ca²⁺ oscillations recorded. F/F₀ represents fluorescent intensity of Ca²⁺ dye relative to baseline.

Figure 5

Expression of wild type and I489F mutant PLC ζ C2 domains as MBP-tagged recombinant proteins

(A) Schematic representation of the MBP fusion protein PLC ζ C2 domains with numbers denoting their amino acid coordinates. (B) SDS-PAGE of affinity-purified recombinant MBP-tagged PLC ζ C2 domains (1 μ g) analysed by 10% SDS-PAGE and Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using the penta-His antibody (1:5,000 dilution); (right panel).

Figure 6

CD analysis of MBP and MBP-tagged PLC ζ C2 fusion proteins

(A) CD spectra were recorded at 4 °C for MBP (black), MBP-C2 ζ ^{WT} (blue) and MBP-C2 ζ ^{I489F} (red). (B) Thermal stability was monitored at 221nm upon heating. In contrast to MBP, the fusion proteins precipitated at ~ 63 °C. Dashed lines represent best fits assuming a two-state unfolding mechanism and an ellipticity of the unfolded state common with MBP.

Figure 7

I489F mutation does not affect the *in vitro* enzyme specific activity and the Ca²⁺ sensitivity of PLC ζ

(A) PIP₂ hydrolysis enzyme activity of MBP-PLC ζ ^{WT} and MBP-PLC ζ ^{I489F} obtained with the standard [³H]PIP₂ hydrolysis assay. Values are means \pm S.E.M. (n=4), using two different preparations of recombinant protein and each experiment was performed in duplicate. (B) Effect of various [Ca²⁺] on the normalized activity of MBP-PLC ζ ^{WT} and MBP-PLC ζ ^{I489F} recombinant proteins. For these assays, values are \pm S.E.M. (n=4), using two different batches of recombinant proteins and with each experiment performed in duplicate (see Table 2).

Figure 8

I489F mutation reduces the binding of PLC ζ to PI(3)P and PI(5)P containing liposomes

Liposome ‘pull-down assays’ of MBP-tagged PLC ζ^{WT} and PLC ζ^{I489F} proteins. Unilamellar liposomes containing either PIP₂ (1%), PI(3)P (5%), or PI(5)P (5%) were incubated with PLC ζ recombinant proteins. Following liposome centrifugation, both the supernatant (s) and liposome pellet (p) were subjected to SDS-PAGE and Coomassie Brilliant Blue staining.

Figure 9

Quantitative analysis suggest that I489F mutation reduces ~50% the binding of PLC ζ to PI(3)P and PI(5)P containing liposomes

Normalized binding of MBP-PLC ζ^{WT} and MBP-PLC ζ^{I489F} recombinant proteins to unilamellar liposomes containing (A) 1% PIP₂, (B) 5% PI(3)P and (C) 5% PI(5)P. Following centrifugation, the supernatants were assayed for their ability to hydrolyse PIP₂ *in vitro*, using the standard [³H]PIP₂ hydrolysis assay, (n=4±SEM, using two different preparations of recombinant protein). Based on the % of the PIP₂ hydrolytic activity pre- and post- liposome binding the relative binding of each PLC ζ protein to the liposomes was determined. Significant statistical differences (asterisks) were calculated by an unpaired Student’s t-test; ****P* < 0.0005, (GraphPad, Prism 5).

Figure 10

Schematic illustration of a proposed intracellular targeting mechanism of PLC ζ

Our study suggests that association of PLC ζ with a specific vesicular membrane may be mediated by interaction of the C2 domain with PI(3)P, PI(5)P or an as yet unidentified membrane protein. Then, association of PLC ζ with the negatively-charged PIP₂ involves electrostatic interactions with the positively-charged 1st EF-hand domain and the XY-linker region. The catalytic XY domain subsequently proceeds with the enzymatic cleavage of PIP₂. The high Ca²⁺ sensitivity of the enzyme is conferred by the EF hand domain enabling PLC ζ to be active at resting nanomolar Ca²⁺ levels (Figure modified from [10]).

TABLES

Table 1

Construct	Pipette cRNA concentration (μg/μl)	Number of eggs injected	Number of eggs oscillating	Mean number of spikes 1 st hr of spiking	Average expression in 1 st hr of spiking (cps)
PLCζ ^{WT} -LUC	0.015	20	19/20	4 ± 0.290	0.06 ± 0.007
PLCζ ^{I489F} -LUC	0.015	19	1/19	0.15 ± 0.016	0.06 ± 0.004
PLCζ ^{I489F} -LUC	0.1	16	15/16	2.3 ± 0.250	0.74 ± 0.038

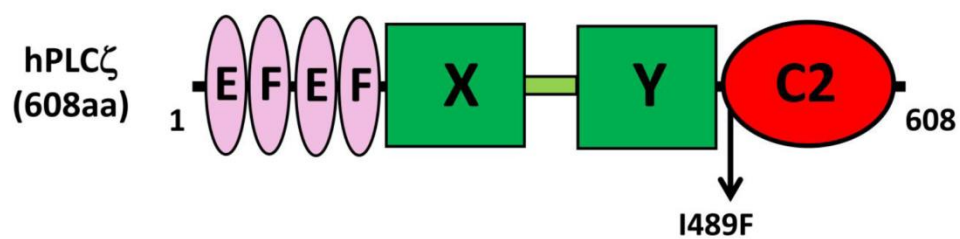
Table 2

PLCζ protein	PIP ₂ hydrolysis enzyme activity (nmol/min/mg)	Ca ²⁺ dependence EC ₅₀ (nM)	<i>K_m</i> (μM)
PLCζ ^{WT}	960±43	66	81
PLCζ ^{I489F}	953±49	60	93

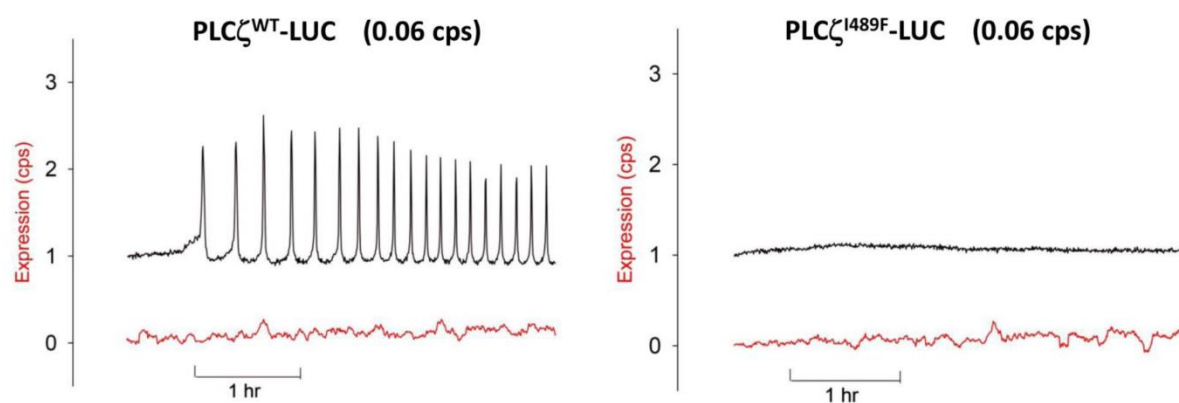
FIGURES

Figure 1

A



B



C

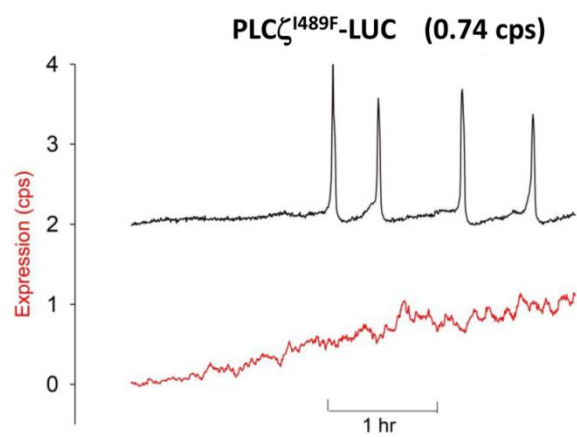


Figure 2

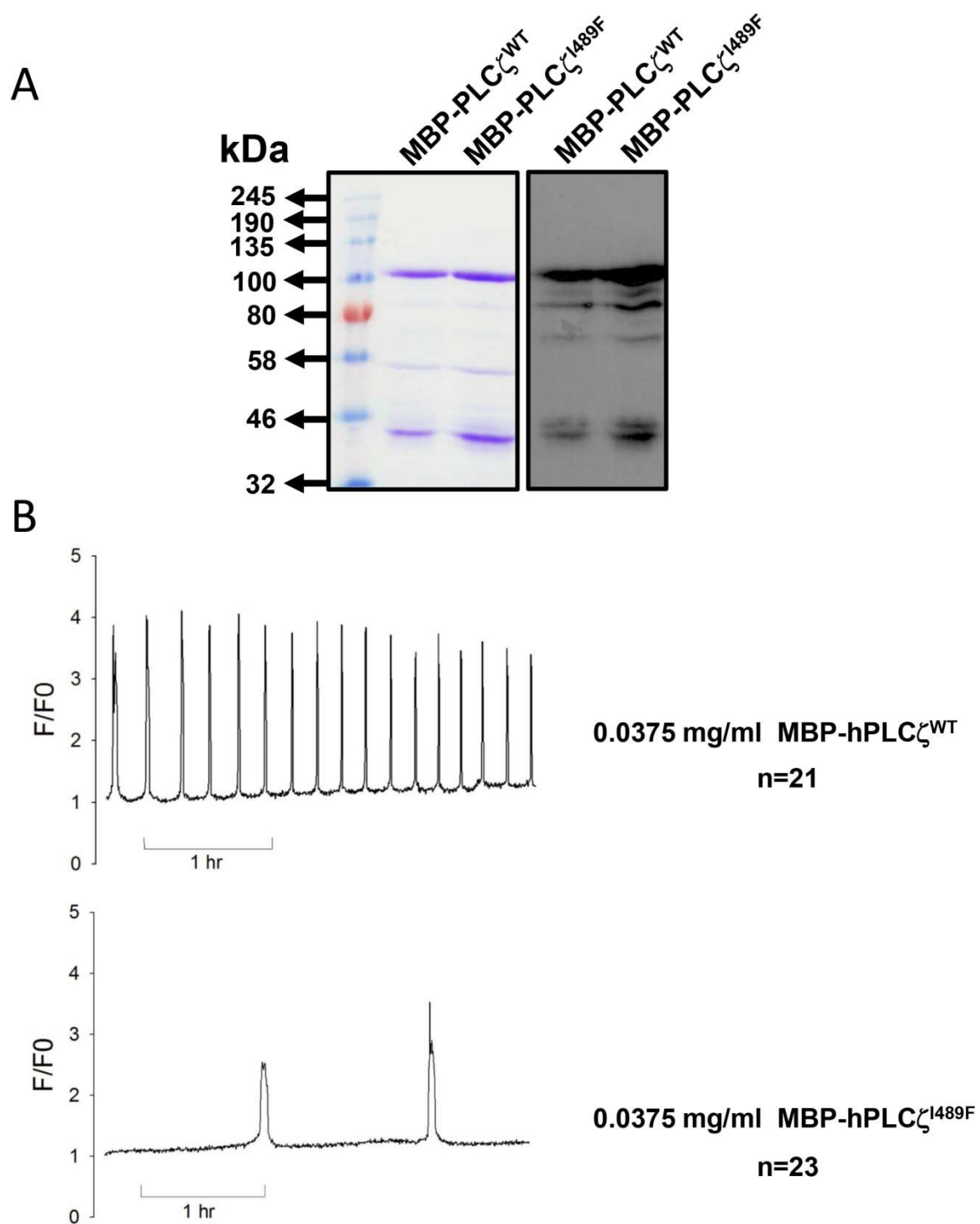


Figure 3

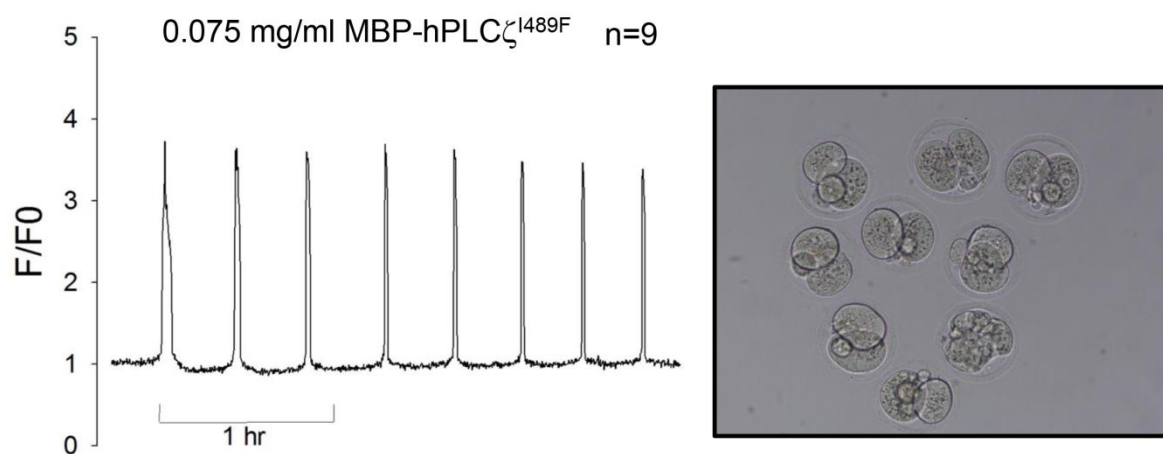


Figure 4

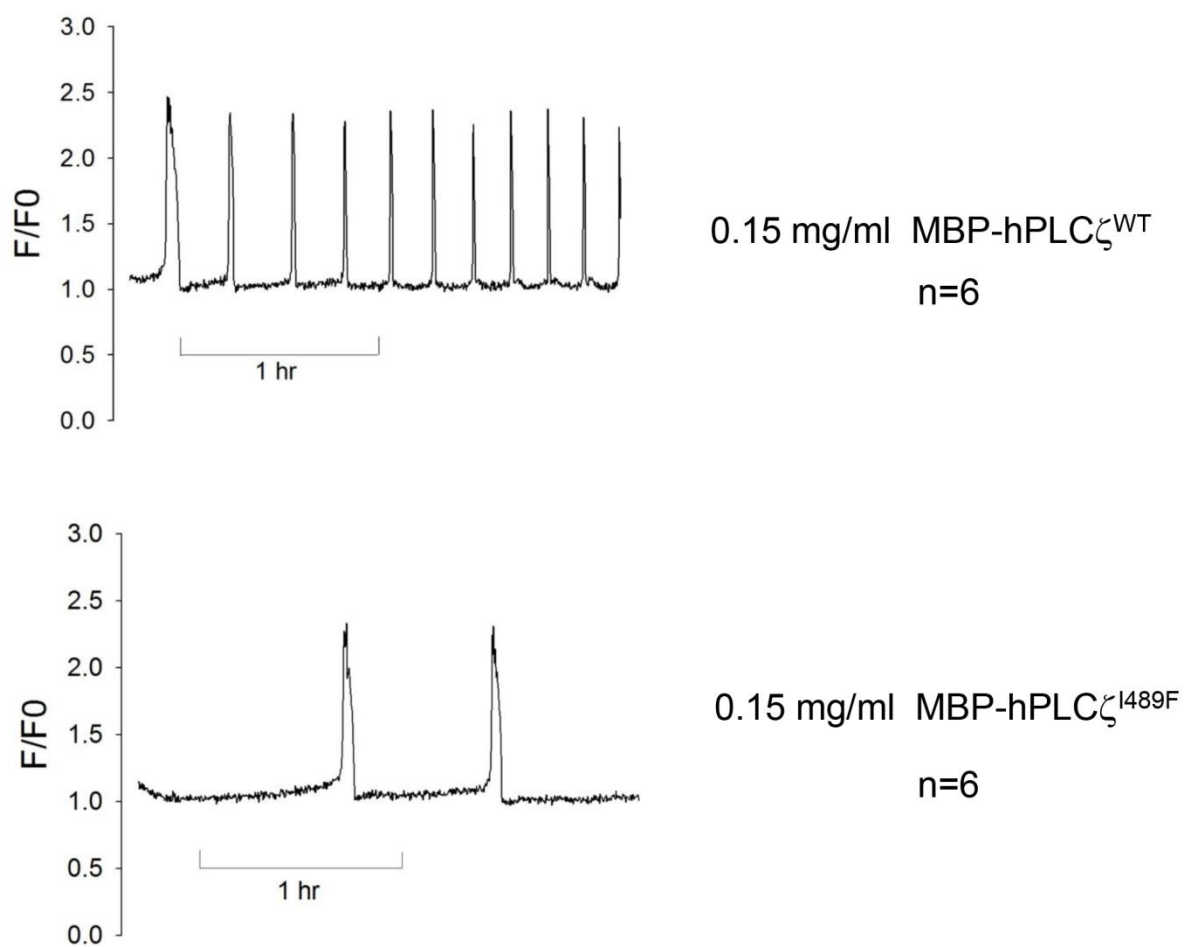


Figure 5

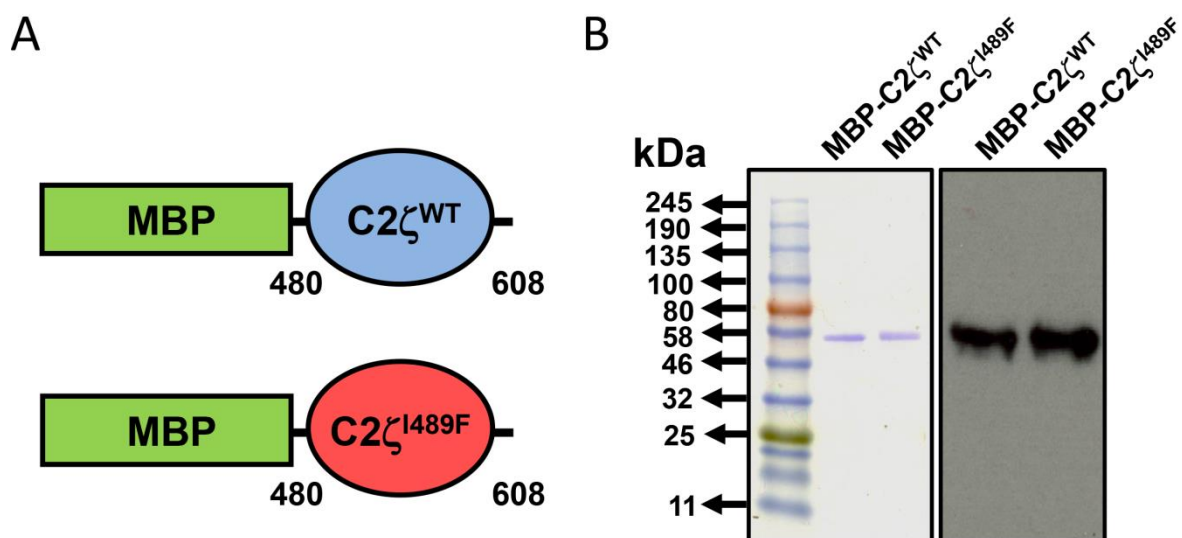


Figure 6

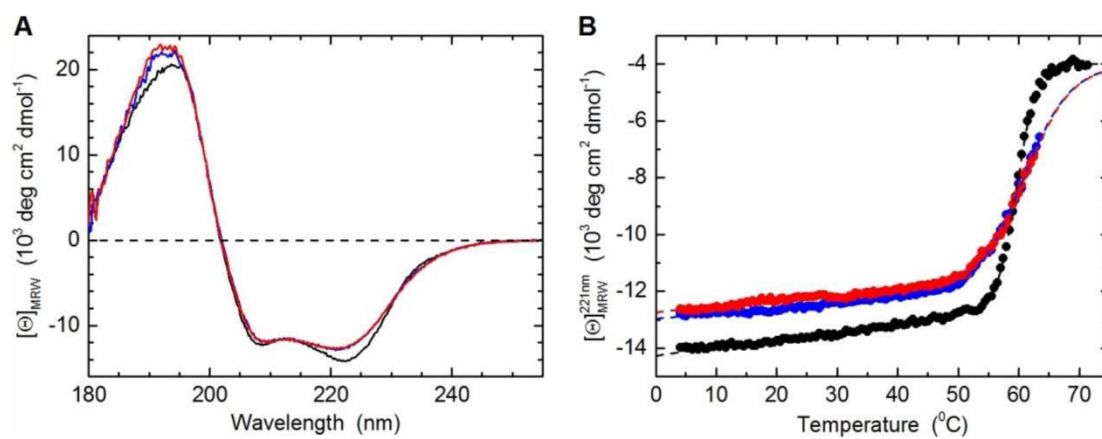


Figure 7

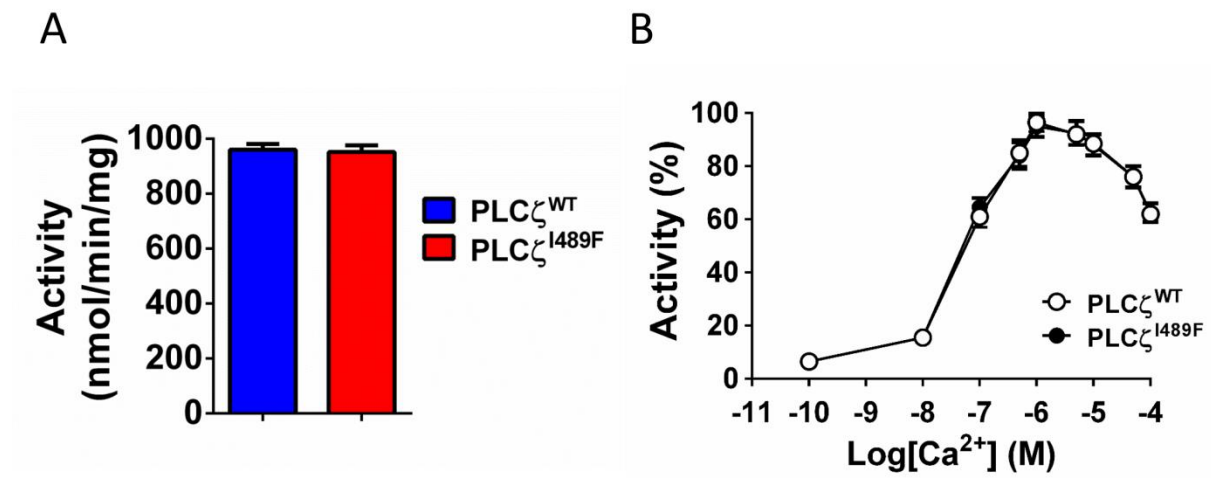


Figure 8

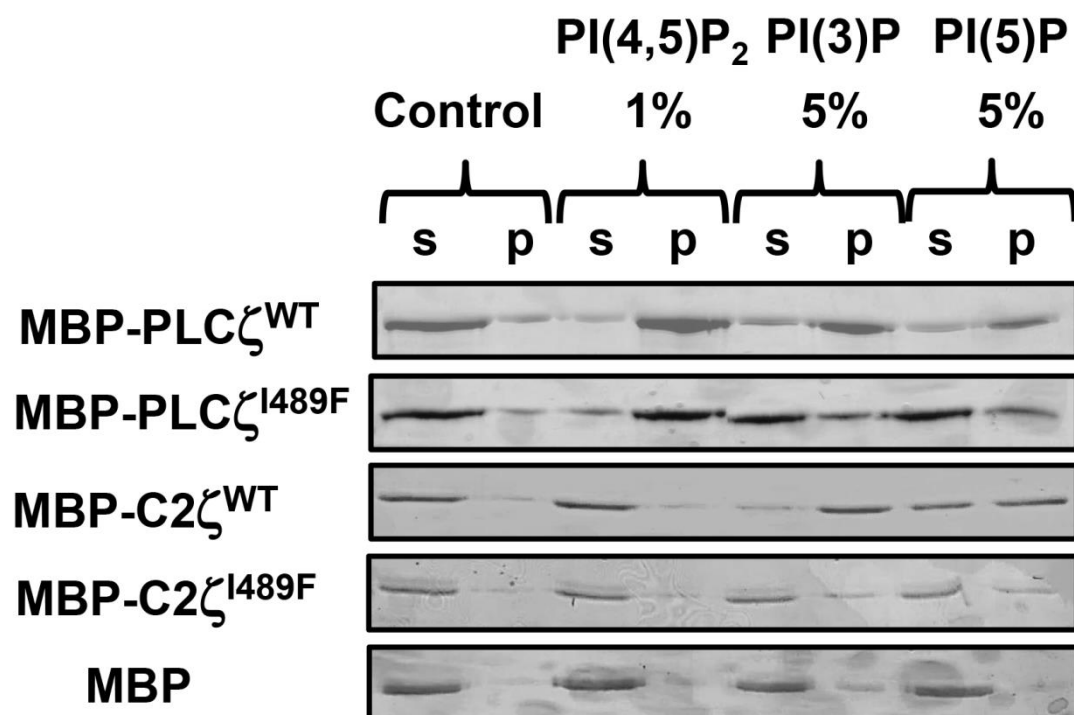


Figure 9

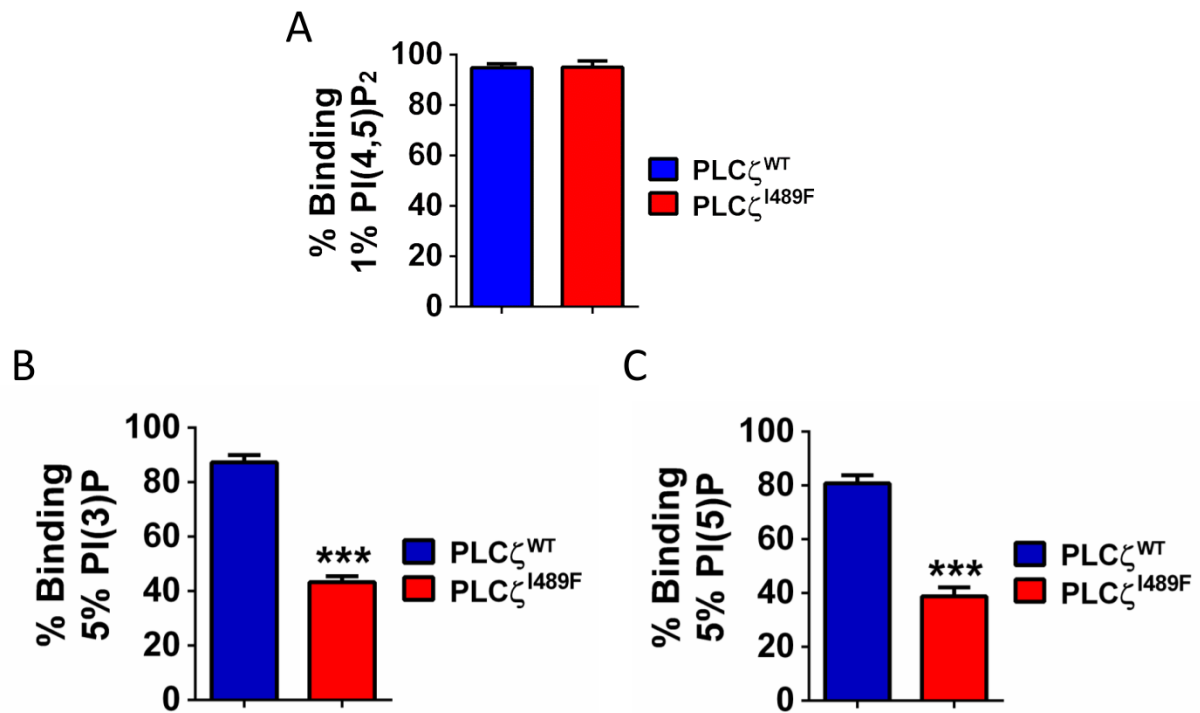


Figure 10

